Novel chemical probes for the investigation of nonribosomal peptide assembly

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1 General methods

Unless specified otherwise, chemicals were purchased from Sigma Aldrich, Fisher Scientific, Carbosynth and Alfa Aesar and were used without further purification. Dry dichloromethane (DCM), tetrahydrofuran (THF) and \( N,N \)-dimethylformamide (DMF) were purchased from VWR International (AR grade) and dried using solvent towers. Dry methanol and dry pyridine were purchased from Fisher Scientific. Reagent grade dichloromethane, ethyl acetate, methanol, cyclohexane, toluene, diethylether, isopropanol, chloroform and tetrahydrofuran were purchased from Fisher Scientific.

Analytical thin-layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel 60 (F254, Merck) and visualized under ultra-violet light (short and long-wave) and using potassium permanganate (KMnO4), vanillin or ninhydrin stains. Silica gel was purchased from Sigma Aldrich (Tech Grade, pore size 60 Å, 230-400 mesh).

Infra-red spectra were recorded on a Perkin-Elmer paragon 1000 FT-IR spectrophotometer. Absorption maxima (\( \nu_{\text{max}} \)) are quoted in wavenumbers (\( \text{cm}^{-1} \)) and only structurally significant peaks are quoted.

\( ^1 \text{H} \) and \( ^{13} \text{C} \) NMR spectra were recorded in \( d_4\)-MeOD, CDCl\(_3\) or D\(_2\)O on the following Bruker Avance instruments: DPX-300 300 MHz, DPX-400 400 MHz, DRX-500 500 MHz or Av-600 600 MHz.

High-resolution mass spectra (HRMS) were obtained using electrospray ionization (ESI) on a MaXis UHR-TOF (Bruker Daltonics) or on Bruker MaXis (ESI-HR-MS).

Optical rotations were obtained using an AA-1000 Polarimeter from Optical Activity LTD.

General method I

\( N \)-(2-aminomethyl) acylamides (1.0 eq.) and \( N-Z \) protected amino acids (1.0 eq.) were dissolved in dry THF (40 mL), followed by the addition of \( N,N \)-disopropylethylamine (DIPEA, 2.0 eq.) under argon atmosphere. The reaction mixture was cooled to 0 °C for 15 min before addition of (1-[Bis(dimethylamino)methylene]-\( 1H \)-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) (HATU, 1.3 eq.). The mixture was stirred at 0 °C for 30 min and then overnight at room temperature. The reaction was concentrated to give a yellow powder. The crude product was dissolved in dichloromethane and washed with 1.0 M HCl (10 mL), sat. NaHCO\(_3\) (aq) (10 mL) and brine (10 mL). The organic phase was then separated, dried over MgSO\(_4\) and filtered. The solvent was removed \textit{in vacuo} and the crude product was purified by silica gel chromatography.

General method II

To a solution of the Cbz-protected compound (1.0 eq.) in anhydrous methanol (20 - 50 mL), palladium on carbon (Pd/C, 0.8 eq.) was added under nitrogen atmosphere. The mixture was then degassed and hydrogen gas was bubbled through it. The reaction was stirred at room temperature under a hydrogen atmosphere until completion. Pd/C was removed by filtration through Celite and washed with acetonitrile. The solvent was removed \textit{in vacuo} to obtain the pure final product without further purification.
**General method III**

Triethylamine (2.0 eq.) was added to a solution of benzyl (2-aminoethyl) carbamates 24 (1.0 eq.) in dry dichloromethane (25 mL). The reaction mixture was cooled to 0 °C before acyl chloride (1.3 eq.) was slowly added. The mixture was stirred at 0 °C for 30 min, followed by stirring at room temperature overnight. The reaction was washed with aqueous HCl (1 M, 10 mL), saturated aqueous NaHCO₃ (10 mL) and brine (10 mL). The organic phase was dried over MgSO₄, filtered and concentrated in vacuo. The residue was purified by silica gel chromatography.

**General method IV**

In a three-necked round-bottom-flask, N-Z-protected amino acid (e.g. 42, 1.10 eq.) was dissolved in dry tetrahydrofuran (THF, 10.0 mL) at 0 °C. Oxalyl chloride (1.10 eq.) and dry N,N-dimethylformamide (DMF, 0.27 eq.) were added and the reaction mixture was stirred for 1 h at 0 °C, followed by 10 min at room temperature. N-(2-aminoethyl) acylamide (1.00 eq.) was dissolved in dry THF (20.0 mL) together with anhydrous pyridine (2.30 eq.) in presence of 3 Å molecular sieves. This solution was stirred for 2 h at room temperature and then added dropwise to the first solution containing activated amino acid. The mixture was stirred at room temperature overnight. The solvent was removed in vacuo to afford a yellow brown solid, which was purified by silica gel chromatography.
2 Synthesis and characterisation of chemical probes 3-17

2.1 Synthesis of (S)-N-(2-acetamidoethyl)-2-aminopropanamide (3)

![Reaction Scheme]

Intermediate 21 (benzyl (S)-(1-((2-acetamidoethyl) amino)-1-oxopropan-2-yl) carbamate) was synthesised according to general method I from N-(2-Aminoethyl) acetamide 19 (200 mg, 1.95 mmol), Z-Ala-OH (20, 436 mg, 1.95 mmol), DIPEA (677 µL, 3.90 mmol) and HATU (964 mg, 2.54 mmol). The crude product was purified by column chromatography with a stepwise gradient (from pure EtOAc to EtOAc: methanol 9:1), affording 21 as a white powder (330 mg, 55 %); Rf = 0.17 (MeOH:EtOAc 1:9); \( v_{\text{max}}/\text{cm}^{-1} \): 3290, 1688, 1649, 1560, 1537, 1446, 1372, 1242, 695; \(^1H\) NMR (500 MHz, CD3OD): \( \delta \) 7.37 – 7.25 (5H, m, Ar-H), 5.09 (1H, d, J = 12.0 Hz, CH2Ar), 5.05 (1H, d, J = 12.0 Hz, CH2Ar), 4.05 (1H, t, J = 7.0 Hz, COCHN), 3.28 – 3.22 (4H, m, NHCH2CH2NH), 1.89 (3H, s, COCH3), 1.30 (3H, d, J = 7.0 Hz, CHCH3); \(^{13}C\) NMR (125 MHz, CD3OD): \( \delta \)c 176.1 (CH2CO), 173.7 (COCH), 158.4 (COO), 138.2 (Ar), 129.5 (Ar), 129.1 (Ar), 128.9 (Ar), 67.8 (CH2Ar), 52.3 (COCH), 40.1 (CH3), 39.9 (CH2), 22.7 (CH3CO), 18.3 (CHCH3); HRMS (ESI): calculate for C15H21N3NaO4 [M+Na]^+: 330.1424, found: 330.1423.

Intermediate 21 (300 mg, 0.98 mmol) was then subjected to hydrogenation according to general method II using Pd/C (83.0 mg, 0.78 mmol) to obtain 3 as a white powder (168 mg, 99 %).

\[\text{HO\textsubscript{3}C\textsubscript{N}\textsubscript{3}}\text{NaO}_{4}\text{[M+Na]^+: 330.1424, found: 330.1423.}\]
(CH₃CO), 20.9 (CH₃CH₂)HRMS (ESI): calculated for C₇H₁₇N₃O₂ [M+H]+: 174.1237, found: 174.1237; [α]₀⁺⁺⁺⁺ = +5.24 (c = 0.005, CH₃OH).

2.2 Synthesis of (S)-N-(2-(2-aminopropanamido)ethyl) butyramide (4)

A solution of benzylchloroformate (23, 1.3 mL, 9.0 mmol) in dry dichloromethane (25 mL) was added over 1.5 h to a solution of ethylenediamine (22, 6.0 mL, 90 mmol) in anhydrous dichloromethane (90 mL) at 0 °C under argon atmosphere. The mixture was stirred at 0 °C for 2 h and then washed with brine, dried over MgSO₄, filtered and concentrated in vacuo to afford intermediate 24 (benzyl (2-aminoethyl) carbamate) as a white powder (1.70 g, 95 %); this was directly utilised without further purification. ¹H NMR (500 MHz, CD₃OD): δ 7.35 - 7.26 (5H, m, Ar-H), 5.06 (2H, s, CH₂Ar), 3.19 (2H, t, J = 6.0 Hz, NHC₂H₂), 2.73 (2H, t, J = 6.0 Hz, CH₂NH₂); ¹³C NMR (125 MHz, CD₃OD): δC 159.2 (CO), 138.4 (Ar), 129.6 (Ar), 129.1 (Ar), 128.3 (Ar), 67.6 (CH₂Ar), 41.5 (CH₂NH₂), 43.9 (NHCH₂); LRMS: calculated for C₁₀H₁₅N₂O₂ [M+H]+: 195.2, found: 195.2. NMR data in accordance with those reported in the literature.¹ This compound is also commercially available.

Intermediate 26 (benzyl (2-butyramidoethyl) carbamate) was synthesised using general method III from 24 (1.00 g, 5.15 mmol), butyryl chloride (25, 0.69 µL, 6.70 mmol) and triethylamine (1.45 mL, 10.3 mmol). The crude product was purified by silica gel chromatography with pure EtOAc. 26 was obtained as a white solid (0.75 g, 55 %); Rf = 0.42 (pure EtOAc); vₘₐₓ/cm⁻¹: 3311, 2961, 2872, 1689, 1644, 1543, 1463, 1375, 1274,
1147, 995, 744, 696, 666; \textsuperscript{1}H NMR (500 MHz, CD\textsubscript{3}OD): \(\delta\) 7.37 - 7.24 (5H, m, Ar-H), 5.04 (2H, s, CH\textsubscript{2}Ar), 3.27 - 3.23 (2H, m, CONHCH\textsubscript{3}), 3.22 - 3.17 (2H, m, CH\textsubscript{2}NHCOO), 2.13 (2H, t, \(J = 7.5\), CH\textsubscript{2}CO), 1.58 (2H, sext, \(J = 7.5\), CH\textsubscript{2}CH\textsubscript{2}), 0.90 (3H, t, \(J = 7.5\), CH\textsubscript{3}); \textsuperscript{13}C NMR (125 MHz, CD\textsubscript{3}OD): \(\delta\) 176.6 (CH\textsubscript{2}CONH), 159.1 (NHCOO), 138.4 (Ar), 129.5 (Ar), 129.0 (Ar), 128.9 (Ar), 67.5 (CH\textsubscript{2}Ar), 41.5 (CH\textsubscript{2}NHCOO), 40.4 (CONHCH\textsubscript{2}), 39.1 (CH\textsubscript{2}CO), 20.3 (CH\textsubscript{3}), 14.0 (CH\textsubscript{3}); HRMS (ESI): calculated for C\textsubscript{14}H\textsubscript{20}N\textsubscript{2}NaO\textsubscript{3} [M+Na]+: 287.1366, found: 287.1367.

Intermediate 26 (600 mg, 2.61 mmol) was then subjected to hydrogenation according to \textit{general method II} using Pd/C (222 mg, 2.09 mmol) to obtain 27 (N-(2-aminoethyl) butyramide) as a white solid (396 mg, 99%). \(v_{\text{max}}/\text{cm}^{-1}\): 3289, 2961, 2871, 1639, 1547, 1493, 1342, 1283, 1236, 707, 481; \textsuperscript{1}H NMR (400 MHz, CD\textsubscript{3}OD): \(\delta\) 3.38 (2H, t, \(J = 6.0\) Hz, NHCH\textsubscript{2}), 2.98 (2H, t, \(J = 6.0\) Hz, CH\textsubscript{2}NH\textsubscript{2}), 2.15 (2H, t, \(J = 7.5\) Hz, CH\textsubscript{2}CO), 1.58 (2H, sext, \(J = 7.5\) Hz, CH\textsubscript{2}CH\textsubscript{2}), 0.89 (3H, t, \(J = 7.5\) Hz, CH\textsubscript{3}); \textsuperscript{13}C NMR (125 MHz, CD\textsubscript{3}OD): \(\delta\) C 176.8 (CO), 42.5 (CH\textsubscript{2}NH\textsubscript{2}), 42.0 (CH\textsubscript{2}NHCOO), 39.2 (CH\textsubscript{2}CO), 20.5 (CH\textsubscript{3}), 14.1 (CH\textsubscript{3}); HRMS (ESI): calculated for C\textsubscript{14}H\textsubscript{20}N\textsubscript{2}NaO [M+Na]+: 153.0998, found: 153.0999.

Intermediate 28 (benzyl (S)-(1-((2-butyramidoethyl) amino)-1-oxopropan-2-yl carbamate) was synthesised according to the \textit{general method I} from compound 27 (128 mg, 0.89 mmol), 20 (200 mg, 0.89 mmol), DIPEA (311 \(\mu\)L, 1.78 mmol) and HATU (441 mg, 1.16 mmol). The crude product was purified by column chromatography with a stepwise gradient (pure EtOAc to EtOAc:MeOH 9:1), giving 28 as a white powder (290 mg, 97%); \(R_f\) = 0.32 (EtOAc:MeOH 95:5). \(v_{\text{max}}/\text{cm}^{-1}\): 3287, 1686, 1649, 1563, 1538, 1445, 1239, 697; \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}): \(\delta\) 7.38 - 7.30 (5H, m, Ar-H), 6.82 (1H, br s, NH), 6.16 (1H, br s, NH), 5.34 (1H, d, \(J = 6.0\) Hz, NHCOO), 5.13 (1H, d, \(J = 12.0\) Hz, CH\textsubscript{2}Ar), 5.08 (1H, d, \(J = 12.0\) Hz, CH\textsubscript{2}Ar), 4.18 (1H, quint, \(J = 7.0\) Hz, COCH\textsubscript{NH}), 3.44 - 3.30 (4H, m, NH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}NH), 2.14 (2H, t, \(J = 7.5\) Hz, CH\textsubscript{2}CO), 1.63 (2H, sext, \(J = 7.5\) Hz, CH\textsubscript{2}CH\textsubscript{2}CO), 1.37 (3H, d, \(J = 7.0\) Hz, CH\textsubscript{3}CH\textsubscript{3}), 0.92 (3H, t, \(J = 7.5\) Hz, CH\textsubscript{3}); \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}): \(\delta\) C 174.3 (CH\textsubscript{2}CO), 173.3 (COCH), 156.0 (COO), 136.1 (Ar), 128.6 (Ar), 128.3 (Ar), 128.1 (Ar), 67.1 (CH\textsubscript{2}Ar), 50.8 (COCH), 40.2 (CH\textsubscript{2}), 39.5 (CH\textsubscript{2}), 38.5 (CH\textsubscript{2}CO), 19.1 (CH\textsubscript{3}CH\textsubscript{2}), 18.5 (CH\textsubscript{3}CH\textsubscript{2}), 13.7 (CH\textsubscript{3}CH\textsubscript{2}); HRMS (ESI): calculated for C\textsubscript{17}H\textsubscript{21}N\textsubscript{2}NaO\textsubscript{4} [M+Na]+: 358.1737, found: 358.1736.

Intermediate 28 (280 mg, 0.83 mmol) was then subjected to hydrogenation according to \textit{general method II} using Pd/C (71.0 mg, 0.67 mmol) to obtain the final compound 4 as a white powder (138 mg, 83%).

\(v_{\text{max}}/\text{cm}^{-1}\): 3283, 2965, 1636, 1541, 1446, 1236, 686; \textsuperscript{1}H NMR (400 MHz, CD\textsubscript{3}OD): \(\delta\) 3.42 - 3.36 (1H, m, COCH), 3.33 - 3.30 (4H, m, NH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}NH), 2.18 (2H, t, \(J = 7.5\) Hz, CH\textsubscript{2}CO), 1.65 (2H, sext, \(J = 7.5\) Hz, CH\textsubscript{2}CH\textsubscript{2}CO), 1.27 (3H, d, \(J = 7.0\) Hz, CH\textsubscript{3}CH\textsubscript{3}), 0.96 (3H, t, \(J = 7.5\) Hz, CH\textsubscript{2}CH\textsubscript{3}); \textsuperscript{13}C NMR (100 MHz, CD\textsubscript{3}OD): \(\delta\) C...
178.8 (COCHNH₂), 176.5 (CH₂CO), 51.6 (COCHNH₂), 40.1 (CH₂), 39.9 (CH₂), 39.1 (CH₂CO), 21.5 (CH₃CH), 20.3 (CH₃CH₂) 14.1 (CH₃CH₃); HRMS (ESI): calculated for C₉H₂₀N₃O₂ [M+H]+: 202.1550, found: 202.1552; [α]D₂₇ = +1.04 (c = 0.005, CH₃OH).

2.3 Synthesis of (S)-N-(2-(2-aminopropanamido)ethyl) heptanamide (5)

Intermediate 30 (benzyl (2-heptanamidoethyl) carbamate) was synthesised according to general method III from 24 (1.00 g, 5.15 mmol), heptanoyl chloride (29, 0.87 mL, 6.70 mmol) and triethylamine (1.22 mL, 10.3 mmol). The crude product was purified by silica gel chromatography with EtOAc: cyclohexane (3:1) to give 30 as a white solid (1.26 g, 80%); Rf = 0.3 (EtOAc : cyclohexane 3:1); νmax/cm⁻¹: 3316, 2926, 2854, 1692, 1642, 1545, 1277, 995, 710, 710; ¹H NMR (600 MHz, CDCl₃): δH 7.26-7.20 (5H, m, Ar-H), 5.87 (1H, br s, CONH₂), 5.08 (1H, br s, CH₂Ar), 4.99 (2H, s, CH₂Ar), 3.30 - 3.25 (2H, m, NHCH₂CH₂NH), 3.25 - 3.20 (2H, m, NHCH₃(CH₂)NH), 2.03 (2H, t, J = 7.5 Hz, CH₂CO), 1.48 (2H, quint, J = 7.5 Hz, CH₃CH₂CO), 1.21 - 1.14 (6H, m, CH₂), 0.78 - 0.75 (3H, m, CH₃); ¹³C NMR (150 MHz, CD₃OD): δC 174.1 (CH₂CO), 157.2 (NHCO), 136.3 (Ar), 128.5 (Ar), 128.2 (Ar), 128.1 (Ar), 66.9 (CH₂Ar), 66.9 (CONHCH₂), 41.0 (CONHCH₂), 40.2 (CH₂CH₂NH), 36.7 (CH₂CO), 31.5 (CH₃), 28.9 (CH₃), 25.6 (CH₂CH₂CO), 22.5 (CH₂), 14.0 (CH₃); HRMS (ESI): calculated for C₁₇H₂₆N₄NaO₃ [M+Na⁺]: 329.1836, found: 329.1835.

Intermediate 30 (650 mg, 2.39 mmol) was subjected to hydrogenation according to general method II using Pd/C (202 mg, 1.91 mmol) to afford compound 31 (N-(2-aminoethyl) heptanamide) as a white solid (406 mg, 99%).
Intermediate 32 (benzyl (S)-1-((2-heptanamidoethyl) amino)-1-oxopropan-2-yl) carbamate) was synthesised according to the general method I from compound 31 (144 mg, 0.84 mmol), 20 (188 mg, 0.84 mmol), DIPEA (293 µL, 1.68 mmol) and HATU (415 mg, 1.09 mmol). The crude product was purified by column chromatography with a stepwise gradient (from pure EtOAc to EtOAc:MeOH 9:1), giving 32 as a white powder (250 mg, 66 %); \( R_f = 0.32 \) (EtOAc:MeOH 9:1); \( \nu_{\text{max}}/\text{cm}^{-1} = 3288, 2929, 2857, 1667, 1650, 1537, 1445, 1238, 1080, 696; ^1H\ NMR (500 MHz, CD_3OD): \delta = 7.36 - 7.26 (5H, m, Ar-H), 5.09 (1H, d, J = 12.0 Hz, CH_2Ar), 5.05 (1H, d, J = 12.0 Hz, CH_2Ar), 4.04 (1H, q, J = 7.0 Hz, COCH_2NH), 3.29 - 3.22 (4H, m, NHCH_2CH_2NH), 2.14 (2H, t, J = 7.5 Hz, CH_2CO), 1.60 - 1.52 (2H, m, CH_2CH_2CO), 1.30 (3H, d, J = 7.0 Hz, CHCH_3), 1.29 - 1.25 (6H, m, CH_3), 0.89 - 0.85 (3H, m, CH_2CH_3); ^13C\ NMR (125 MHz, CD_3OD): \delta = 176.7 (CH_2CO), 176.1 (COCH_2), 158.4 (COO), 138.2 (Ar), 129.5 (Ar), 129.1 (Ar), 128.9 (Ar), 67.8 (CH_2Ar), 52.3 (COCH_2), 40.2 (CH_3), 39.9 (CH_3), 37.3 (CH_2CO), 32.7 (CH_3), 30.1 (CH_3), 26.9 (CH_2CH_2CO), 23.6 (CH_3), 18.3 (CHCH_3), 14.4 (CH_3CH_2); HRMS (ESI): calculated for C_{20}H_{31}N_3NaO_4 [M+Na]^+: 440.2207, found: 440.2213.

Intermediate 32 (240 mg, 0.64 mmol) was subjected to hydrogenation according to general method II using Pd/C (54.0 mg, 0.51 mmol) to obtain the final compound 5 as a white powder (152 mg, 62 %).
2.4 Synthesis of (S)-N-(2-(2-aminopropanamido)ethyl) decanamide (6)

Intermediate 34 (benzyl (2-decanamidoethyl) carbamate) was synthesised according to general method III from 24 (1.00 g, 5.15 mmol) and decanoyl chloride (33, 1.38 mL, 6.70 mmol). The crude product was purified by column chromatography with EtOAc : cyclohexane (1:1) yielding 34 as a white solid (1.05 g, 59 %); Rf = 0.22 (EtOAc : cyclohexane 1:1); ν_max/cm⁻¹: 3321, 3305, 2916, 2849, 1688, 1638, 1545, 1446, 1276, 1246, 1147, 997, 727, 667; ¹H NMR (500 MHz, CD3OD): δ_H = 7.34 – 7.25 (5H, m, Ar-H), 5.04 (2H, s, COOCH₂), 3.24 (2H, t, J = 5.5 Hz, CONHCH₂), 3.18 – 3.21 (2H, m, CH₂NHCOCO), 2.13 (2H, t, J = 7.5 Hz, CH₂CO), 1.60 – 1.52 (2H, m, CH₂CH₂CO), 1.33–1.23 (12H, m, CH₂); ¹³C NMR (125 MHz, CD3OD): δ_C = 176.9 (CH₂CONH), 159.2 (NHCOCH), 138.5 (Ar), 129.6 (Ar), 129.1 (Ar), 129.0 (Ar), 67.6 (OCH₂Ph), 41.6 (CH₂NHCOCO), 40.5 (CONHCH₂), 37.3 (CH₂CO), 33.2 (CH₃), 30.7 (CH₃), 30.6 (CH₃), 30.5 (CH₃), 27.1 (CH₂), 23.9 (CH₂), 14.2 (CH₃); HRMS [ESI]: calculated for C₂₀H₃₂N₂NaO₃ [M+Na⁺]: 371.2305, found: 371.2305.

Intermediate 34 (660 mg, 1.90 mmol) was then subjected to hydrogenation according to general method II using Pd/C (161 mg, 1.52 mmol) to obtain the compound 35 (N-(2-aminooethyl) decanamide) as a white powder (160 mg, 98 %); Rf = 0.2 (CH₂Cl₂ : MeOH 3:7); ν_max/cm⁻¹: 3285, 2918, 2849, 1635, 1552, 1466, 967, 927, 719; ¹H NMR (400 MHz, CD3OD): δ_H = 3.27 (2H, t, J = 6.0 Hz, NHCH₂), 2.78 (2H, t, J = 6.5 Hz, CH₂NH₂), 2.18 (2H, t, J = 7.5 Hz, CH₂CO), 1.64 – 1.54 (2H, m, CH₂CH₂CO), 1.35 – 1.24 (12H, m, CH₂); ¹³C NMR (100 MHz, CD3OD): δ_C = 173.2 (CO), 41.9 (NHCH₂), 41.6 (CH₂NH₂), 37.0 (CH₂CO), 33.9 (CH₃), 29.6 (CH₂), 29.5(CH₃), 29.5 (CH₂), 26.4 (CH₂CH₂CO), 25.9 (CH₃), 22.8 (CH₂), 14.3 (CH₃); HRMS [ESI]: calculated for C₁₅H₂₉N₂O [M+H⁺]: 215.2118, found: 215.2121.
Intermediate 36 (benzyl (5)-(1-((2-decananamidoethyl) amino)-1-oxopropan-2-yl) carbamate) was synthesised according to the general method I from compound 35 (200 mg, 0.78 mmol), 20 (174 mg, 0.78 mmol), DIPEA (272 µL, 1.56 mmol) and HATU (388 mg, 1.02 mmol). The crude product was purified by silica gel chromatography with a stepwise gradient (EtOAc: MeOH 99:1), affording 36 as a white powder (280 mg, 86 %); Rf = 0.3 (EtOAc: MeOH 99:1).

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$H 7.38 – 7.29 (5H, m, Ar-H), 6.85 (1H, br s, NH), 6.16 (1H, br s, NH), 5.39 (1H, d, J = 7.0 Hz, NHCOO), 5.13 (1H, d, J = 12.0 Hz, CH$_3$Ar), 5.07 (1H, d, J = 12.0 Hz, CH$_2$Ar), 4.18 (1H, quint, J = 7.0 Hz, COCHNH), 3.44 – 3.28 (4H, m, NHCH$_2$CH$_2$NH), 2.18 – 2.11 (2H, t, J = 7.5 Hz, CH$_2$CO), 1.64 – 1.54 (2H, m, CH$_2$CH$_2$CO), 1.37 (3H, d, J = 7.0 Hz, CHCH$_3$), 1.32 – 1.22 (12H, m, CH$_3$), 0.90 – 0.85 (3H, m, CH$_3$CH$_3$); $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$C 174.7 (CH$_2$CO), 173.4 (COCH), 156.1 (COO), 136.3 (Ar), 128.7 (Ar), 128.4 (Ar), 128.2 (Ar), 67.2 (CH$_3$Ar), 50.9 (COCH), 40.4 (CH$_3$), 39.7 (CH$_3$), 36.8 (CH$_2$CO), 31.9 (CH$_3$), 29.6 (CH$_3$), 29.5 (CH$_3$), 29.4 (CH$_3$), 25.8 (CH$_2$CH$_2$CO), 22.8 (CH$_3$), 18.7 (CHCH$_3$), 14.3 (CH$_3$CH$_3$); HRMS (ESI): calculated for C$_{22}$H$_{34}$N$_3$NaO$_4$ [M+Na]$^+$: 442.2676, found: 442.2678.

Intermediate 36 (260 mg, 0.62 mmol) was then subjected to hydrogenation according to general method II using Pd/C (53.0 mg, 0.50 mmol) to obtain the final compound 6 as a white powder (170 mg, 96 %).

$\nu$ max/cm$^{-1}$: 3298, 2918, 2850, 1637, 1552, 1445, 1241, 937, 721; $^1$H NMR (400 MHz, CD$_2$OD): $\delta$H 3.40 (1H, m, COCH), 3.33 – 3.28 (4H, m, NHCH$_2$CH$_2$NH), 2.20 (2H, t, J = 7.5 Hz, CH$_2$CO), 1.69 – 1.55 (2H, m, CH$_2$CH$_2$CO), 1.39 – 1.29 (12H, m, CH$_3$), 1.28 (3H, d, J = 7.0 Hz, CHCH$_3$), 0.99 – 0.86 (3H, m, CH$_3$CH$_2$); $^{13}$C NMR (100 MHz, CD$_2$OD): $\delta$C 178.8 (COCH$_2$N$_2$), 176.7 (CH$_2$CO), 51.6 (COCH$_2$N$_2$), 40.1 (CH$_3$), 39.9 (CH$_3$), 37.2 (CH$_2$CO), 33.0 (CH$_3$), 30.6 (CH$_3$), 30.5 (CH$_3$), 30.4 (CH$_3$), 30.3 (CH$_3$), 26.9 (CH$_2$CH$_2$CO), 23.7 (CH$_3$), 21.5 (CH$_3$CH$_2$), 14.4 (CH$_3$CH$_3$); HRMS (ESI): calculated for C$_{15}$H$_{22}$N$_2$O$_2$ [M+H]$^+$: 286.2489, found: 286.2488; $[\alpha]_D^{28}$ = +3.49 (c = 0.0045, CH$_3$OH).

### 2.5 Synthesis of (S)-2-amino-N-(2-butyramidoethyl)-3-methylbutanamide (7)

![Scheme 55: Preparation of (S)-2-amino-N-(2-butyramidoethyl)-3-methylbutanamide (7).](image-url)
Intermediate 38 (benzyl (S)-(1-((2-butylamidoethyl)amino)-3-methyl-1-oxobutan-2-yl)carbamate) was synthesised according to the general method I from amine 27 (52 mg, 0.40 mmol), Z-Val-OH (37, 100 mg, 0.40 mmol), DIPEA (139 μL, 0.80 mmol) and HATU (198 mg, 0.52 mmol). The crude product was purified by column chromatography with an isocratic elution of EtOAc : MeOH (98:2), affording 38 as a white powder (140 mg, 91%); Rf = 0.32 (EtOAc : MeOH 98:2); νmax/cm⁻¹:3292, 3919, 2851, 1720, 1689, 1646, 1248, 1138, 732; ¹H NMR (500 MHz, CD3OD): δH 7.36 – 7.24 (5H, m, Ar-H), 5.09 (1H, d, J = 12.5 Hz, CH2Ar), 5.06 (1H, d, J = 12.5 Hz, CH2Ar), 3.83 (1H, d, J = 7.0 , COCHNH), 3.28 – 3.23 (4H, m, NHCH2CH2NH), 2.12 (2H, t, J = 7.5 Hz, CH2CO), 2.06 – 1.97 (1H, m, CH(CH3)2), 1.59 (2H, sext, J = 7.5 Hz, CH2CH2), 0.93 – 0.87 (9H, m, CH3); ¹³C NMR (125 MHz, CD3OD): δC 176.5 (CH2CO), 174.73 (COCHNH), 158.7 (COO), 138.2 (Ar), 129.5 (Ar), 129.1 (Ar), 128.9 (Ar), 67.8 (CH2Ar), 62.5 (COCHNH), 40.0 (CH3), 39.9 (CH3), 39.1 (CH3CO), 31.8 (CH(CH3)2), 20.3 (CH3CH2), 19.8 (CH(CH3)2), 18.6 (CH(CH3)2), 14.1 (CH3CH3); HRMS (ESI): calculated for C19H30N3O4 [M+Na⁺]: 386.2050, found: 386.2058.

Intermediate 38 (130 mg, 0.36 mmol) was then subjected to hydrogenation according to general method II using Pd/C (30 mg, 0.29 mmol) to obtain the final compound 7 as a white powder (82 mg, 99%).

2.6 Synthesis of (S)-N-(2(2-amino-3-methylbutanamido)ethyl)heptanamide (8)

Scheme 65: Preparation of (S)-N-(2(2-amino-3-methylbutanamido)ethyl)heptanamide (8).
Intermediate 39 (benzyl (S)-(1-((2-heptanamidooethyl)amino)-3-methyl-1-oxobutan-2-yl)carbamate) was synthesized according to the general method I from compound 31 (74.0 mg, 0.43 mmol), 37 (141 mg, 0.56 mmol), DIPEA (225 µL, 1.29 mmol) and HATU (213 mg, 0.56 mmol). The crude product was purified by column chromatography with an isocratic elution of EtOAc, affording 39 as a white powder (123 mg, 71%); Rf = 0.3 (EtOAc); v_max/cm⁻¹: 3290, 2919, 2851, 1687, 1641, 1536, 1245, 1139, 1039, 677; ¹H NMR (500 MHz, CDCl₃): δH 7.33 - 7.29 (5H, m, Ar-H), 6.94 (1H, br s, NH), 6.32 (1H, br s, NH), 5.48 (1H, m, NH), 5.48 (1H, d, J = 12.0 Hz, CH₂Ar), 5.12 (1H, d, J = 12.0 Hz, CH₂Ar), 4.01 – 3.93 (1H, m, COCHNH), 3.43 – 3.26 (4H, m, NHCH₂CH₂NH), 2.19 - 2.05 (3H, m, CH₂CO, CH(CH₃)₂), 1.64 – 1.52 (2H, m, CH₂CH₂CO), 1.34 – 1.19 (6H, m, CH₂), 0.95 (3H, d, J = 7.0 Hz, CH(CH₃)₂), 0.92 – 0.80 (6H, m, CH₂CH₂, CH(CH₃)₂); ¹³C NMR (100 MHz, CDCl₃): δC 164.6 (COCHNH₂), 172.5 (CH₂CO), 156.6 (COO), 136.3 (Ar), 128.7 (Ar), 128.4 (Ar), 128.1 (Ar), 67.2 (CH₂Ar), 60.8 (COCHNH), 40.2 (CH₃), 39.8 (CH₂), 36.8 (CH₂CO), 31.6 (CH₂), 31.0 (CH(CH₃)₂), 29.1 (CH₃), 25.8 (CH₂CH₂CO), 22.6 (CH₂), 19.4 (CH(CH₃)₂), 17.9 (CH(CH₃)₂), 14.2 (CH₂CH₂); HRMS (ESI): calculated for C₂₂H₃₅N₇O₄ [M+Na]⁺: 428.2520, found: 428.2520.

Intermediate 39 (89 mg, 0.22 mmol) was subjected to hydrogenation according to general method II using Pd/C (19 mg, 0.18 mmol) to obtain the final compound 8 as a white powder (57 mg, 97%).

2.7 Synthesis of [(S)-N-(2-(2-amino-3-hydroxypropanamido)ethyl)heptanamide (9)]

Scheme 75: Preparation of (S)-N-(2-(2-amino-3-hydroxypropanamido)ethyl)heptanamide (9).
Intermediate 41 (benzyl (S)-(1-((2-heptanamidoethy1)amino)-3-hydroxy-1-oxopropan-2-yl)carbamate) was synthesised according to the general method I from compound 31 (360 mg, 2.09 mmol), Z-L-Ser(Bzl)-OH (40, 500 mg, 2.09 mmol), DIPEA (728 μL, 4.18 mmol) and HATU (1.00 g, 2.71 mmol). The crude product was purified by column chromatography with an isocratic elution of EtOAc: petroleum ether (3:1), affording 41 as a white powder (498 mg, 60 %); Rf = 0.3 (EtOAc: petroleum ether 3:1); \( \nu_{\text{max}}/\text{cm}^{-1} \): 3288, 2927, 2858, 1688, 1640, 1537, 1451, 1237, 1027, 732, 693; \(^1\)H NMR (500 MHz, CDCl\(_3\)): \( \delta \_H \) 7.30 - 7.18 (10H, m, Ar-H), 6.82 (1H, br s, NH), 5.97 (1H, br s, NH), 5.64 – 5.54 (1H, m, NH), 5.07 (1H, d, \( J = 12.0 \) Hz, COOCH\(_2\)H), 5.03 (1H, d, \( J = 12.0 \) Hz, COOCH\(_2\)H), 4.45 (2H, m, \( J = 12.0 \) Hz, CH\(_2\)OCH\(_2\)H), 4.24 (1H, s, COCH), 3.89 – 3.79 (1H, m, CH\(_2\)CH\(_2\)), 3.52 (1H, dd, \( J = 9.5 \), 3.5 Hz, CHCH\(_2\)), 3.36 – 3.22 (4H, m, NH\(_2\)CH\(_2\)CH\(_2\)NH), 2.01 (2H, t, \( J = 7.5 \) Hz, CH\(_2\)CO), 1.53 – 1.45 (2H, m, CH\(_2\)CH\(_2\)CO), 1.25 – 1.13 (6H, m, CH\(_2\)), 0.82 – 0.77 (3H, m, CH\(_3\)); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)): \( \delta \_C \) 174.3 (CH\(_2\)CONH), 171.0 (COCH), 156.3 (COO), 137.4 (Ar), 136.1 (Ar), 128.7 (Ar), 128.5 (Ar), 128.3 (Ar), 128.2 (Ar), 127.9 (Ar), 73.6 (CH\(_2\)OCH\(_2\)), 69.8 (CH\(_2\)CH\(_2\)), 67.4 (COOCH\(_2\)), 54.9 (COCH), 40.2 (CH\(_2\)), 39.8 (CH\(_2\)), 36.7 (CH\(_2\)CO), 31.6 (CH\(_2\)), 29.1 (CH\(_3\)CH\(_2\)CH\(_2\)), 25.7 (CH\(_2\)CH\(_2\)CO), 22.6 (CH\(_2\)), 14.2 (CH\(_3\)); HRMS (ESI): calculated for C\(_{29}\)H\(_{37}\)N\(_3\)NaO\(_5\) [M+Na\(^+\)]: 506.2625 found: 506.2625.

To a solution of 41 (51 mg, 0.11 mmol) in anhydrous methanol, Pd/C (112 mg, 1.05 mmol) and ammonium formate (41 mg, 0.63 mmol) were added under argon atmosphere. The reaction was heated at reflux and stirred overnight. The reaction was then filtered through Celite and the solvent was removed in vacuo to obtain the final compound 9 as a white powder (28 mg, 99 %) without further purification.
2.8 Synthesis of N-(2-(2-aminoacetamido)ethyl)butyramide (10)

\[
\begin{align*}
\text{HCOOH} & \xrightarrow{1)} (\text{COCl}_2, \text{DMF (cat.)}, \text{THF}, 0 \degree \text{C}, 2\text{h}) \\
\text{Ph} & \quad \text{O} \\
\text{HO} & \quad \text{N} \\
\text{O} & \quad \text{O} \\
\text{CH}_2\text{NH}_2 & \quad \text{NH}_2 \\
\text{Ph} & \quad \text{Ph} \\
\text{HO} & \quad \text{Ph} \\
\text{O} & \quad \text{O} \\
\text{CH}_2\text{NH}_2 & \quad \text{NH}_2 \\
\end{align*}
\]

\[\text{Pd/C (0.8 e.q.), H}_2, \text{MeOH} \rightarrow \text{O} \quad \text{NH}_2 \quad \text{O} \quad \text{NH}_2 \quad \text{Ph} \quad \text{Ph} \quad \text{HO} \quad \text{O} \quad \text{CH}_2\text{NH}_2 \quad \text{NH}_2 \]

Scheme 8S: Preparation of N-(2-(2-aminoacetamido)ethyl)butyramide (10).

Intermediate 43 (benzyl (2-((2-butyramidoethyl)amino)-2-oxoethyl)carbamate) was synthesised according to general method IV from 27 (316 mg, 2.43 mmol), Z-Gly-OH (42, 559 mg, 2.67 mmol), oxalyl chloride (229 µL, 2.67 mmol), dry DMF (51 µL, 0.66 mmol) and anhydrous pyridine (417 µL, 5.18 mmol). The crude product was purified by silica gel chromatography with an isocratic elution of EtOAc : MeOH (96:4). The product 43 was obtained as a white solid (200 mg, 62 %); \( R_f = 0.18 \) (EtOAc : MeOH 96:4).\(^1\)\(^\text{H NMR}\) (500 MHz, CD\(_3\)OD): \( \delta \_H = 7.38 \sim 7.25 \) (5H, m, Ar-H), 5.09 (2H, s, CH\(_2\)Ph), 3.76 (2H, s, CO\(_2\)NH), 3.30 – 3.20 (4H, m, NH\(_2\)CH\(_2\)CH\(_2\)NH), 2.14 (2H, t, \( J = 7.5 \) Hz, CH\(_2\)CO), 1.60 (2H, sext, \( J = 7.5 \) Hz, CH\(_2\)CH\(_2\)CO), 0.91 (3H, t, \( J = 7.5 \) Hz, CH\(_3\)); \(^{13}\)\(_C\) NMR (125 MHz, CD\(_3\)OD): \( \delta \_C = 176.6 \) (CH\(_2\)CONH), 172.7 (NHCOCH\(_2\)), 159.2 (NHCOO), 138.1 (Ar), 129.5 (Ar), 129.1 (Ar), 129.9 (Ar), 67.9 (CH\(_2\)Ar), 45.1 (CO\(_2\)NH), 40.2 (CH\(_3\)), 39.9 (CH\(_2\)), 39.1 (CH\(_2\)CO), 20.3 (CH\(_3\)CH\(_2\)), 14.1 (CH\(_3\)); HRMS (ESI): calculated for C\(_{16}\)H\(_{23}\)N\(_3\)NaO\(_4\) [M+Na\(^+\)]: 344.1581, found: 344.1583.

Intermediate 43 (280 mg, 0.87 mmol) was subjected to hydrogenation according to general method II using Pd/C (74 mg, 0.70 mmol) to obtain 10 as a white powder (160 mg, 98 %). \( R_f = 0.2 \) (CH\(_2\)Cl\(_2\) : MeOH 3:7).

\[\text{O} \quad \text{NH}_2 \quad \text{NH}_2 \quad \text{NH}_2 \quad \text{NH}_2 \quad \text{O} \quad \text{O} \quad \text{Ph} \quad \text{Ph} \quad \text{O} \quad \text{NH}_2 \quad \text{O} \quad \text{NH}_2 \quad \text{Ph} \quad \text{Ph} \quad \text{HO} \quad \text{O} \quad \text{CH}_2\text{NH}_2 \quad \text{NH}_2 \]

\( v_{\text{max}}/\text{cm}^{-1} : 3279, 2960, 1634, 1544, 1457, 1256, 682; ^1\text{H NMR} \) (500 MHz, CD\(_3\)Cl\(_2\)): \( \delta \_H = 7.69 \) (1H, br s, NH), 6.32 (1H, br s, NH), 3.47 – 3.37 (4H, m, NH\(_2\)CH\(_2\)CH\(_2\)NH), 3.35 (2H, s, CO\(_2\)NH\(_2\)), 2.15 (2H, t, \( J = 7.5 \) Hz, CH\(_2\)CO), 1.65 (2H, sext, \( J = 7.5 \) Hz, CH\(_2\)CH\(_2\)), 1.59 (2H, br s, NH\(_2\)), 0.93 (3H, t, \( J = 7.5 \) Hz, CH\(_3\)); \(^{13}\)\(_C\) NMR (125 MHz, CD\(_3\)Cl\(_2\)): \( \delta \_C = 174.5 \) (CH\(_2\)CONH), 174.0 (CO\(_2\)NH\(_2\)), 44.8 (CO\(_2\)NH\(_2\)), 40.8 (CH\(_3\)), 39.3 (CH\(_2\)), 38.8 (CH\(_2\)CO), 19.3 (CH\(_3\)CH\(_2\)), 13.9 (CH\(_3\)); HRMS (ESI): calculated for C\(_{8}\)H\(_{17}\)N\(_3\)NaO\(_2\) [M+Na\(^+\)]: 210.1213, found: 210.1214.
2.9 Synthesis of N-(2-(2-aminoacetamido)ethyl) heptanamide (11)

\[
\text{HO-} \quad \text{N-} \quad \text{O-} \quad \text{Ph} \quad \xrightarrow{1) \text{(COCl)}_2, \text{DMF (cat.), THF, } 0 \degree \text{C, } 2h} \quad \text{HO-} \quad \text{N-} \quad \text{N-} \quad \text{N-} \quad \text{O-} \quad \text{Ph} \quad \xrightarrow{2) \text{Pd/C (0.8 e.q.), H}_{2}, \text{MeOH}} \quad \text{N-} \quad \text{N-} \quad \text{N-} \quad \text{O-} \quad \text{Ph} \quad \xrightarrow{\text{Pd/C (0.8 e.q.), H}_{2}, \text{MeOH}} \quad \text{N-} \quad \text{N-} \quad \text{N-} \quad \text{O-} \quad \text{Ph}
\]

Scheme 95: Preparation of N-(2-(2-aminoacetamido)ethyl) heptanamide (11).

Intermediate 44 (benzyl (2-(2-heptanamidoethyl)amino)-2-oxoethyl) carbamate) was synthesised according to general method IV from 31 (300 mg, 1.74 mmol), 42 (402 mg, 1.92 mmol), oxalyl chloride (165 µL, 1.92 mmol), dry DMF (36.0 µL, 0.47 mmol) and anhydrous pyridine (299 µL, 3.71 mmol). The crude product was purified by silica gel chromatography with an isocratic elution of EtOAc : MeOH (96:4). The product 44 was obtained as a white solid (520 mg, 82 %); \( R_f = 0.29 \) (EtOAc : MeOH 96:4); \( \nu_{\text{max}}/\text{cm}^{-1} \): 3333, 3285, 2922, 2853, 1689, 1656, 1639, 1541, 1293, 1249, 694; \( ^1\text{H NMR} \) (500 MHz, CD\(_2\)OD): \( \delta \_H \) 7.30 - 7.26 (5H, m, Ar-H), 5.09 (2H, s, CH\(_2\)Ar), 3.72 (2H, s COCH\(_2\)NH), 3.28 - 3.24 (4H, m, NHCH\(_2\)CH\(_2\)NH), 2.15 (2H, t, \( J = 7.5 \) Hz, CH\(_2\)CO), 1.60 - 1.53 (2H, m, CH\(_3\)CH\(_2\)CO), 1.33 - 1.26 (6H, m, CH\(_3\)), 0.89 - 0.86 (3H, m, CH\(_3\)); \( ^{13}\text{C NMR} \) (125 MHz, CD\(_2\)OD): \( \delta_C \) 176.8 (CH\(_2\)CONH), 172.7 (COCH\(_2\)NH), 159.2 (COO), 138.1 (Ar), 129.5 (Ar), 129.1 (Ar), 129.0 (Ar), 68.0 (CH\(_2\)Ar), 45.1 (COCH\(_2\)NH), 40.2 (CH\(_3\)), 39.9 (CH\(_3\)), 37.2 (CH\(_2\)CO), 32.7 (CH\(_2\)CO), 30.1 (CH\(_3\)), 26.9 (CH\(_2\)CH\(_2\)CO), 23.6 (CH\(_2\)CO), 14.4 (CH\(_3\)); HRMS (ESI): calculate for C\(_{19}\)H\(_{29}\)N\(_3\)NaO\(_2\) [M+Na]\(^+\): 386.2050, found: 386.2055.

Intermediate 44 (300 mg, 0.83 mmol) was subjected to hydrogenation according to general method II using Pd/C (70.0 mg, 0.66 mmol) to obtain the final compound 11 as a white powder (120 mg, 63 %). \( R_f = 0.09 \) (CH\(_3\)Cl: MeOH 3:7).

\[
\text{HO-} \quad \text{N-} \quad \text{N-} \quad \text{O-} \quad \text{NH}_2 \quad \xrightarrow{\text{Pd/C (0.8 e.q.), H}_{2}, \text{MeOH}} \quad \text{HO-} \quad \text{N-} \quad \text{N-} \quad \text{O-} \quad \text{NH}_2
\]

\( \nu_{\text{max}}/\text{cm}^{-1} \): 3275, 2921, 2854, 1633, 1557, 1445, 1257, 713; \( ^1\text{H NMR} \) (500 MHz, CD\(_2\)OD): \( \delta \_H \) 3.33 - 3.29 (4H, m, NHCH\(_2\)CH\(_2\)NH), 3.25 (2H, s, COCH\(_2\)NH\(_2\)), 2.19 (2H, t, \( J = 7.5 \) Hz, CH\(_2\)CO), 1.64 - 1.56 (2H, m, CH\(_3\)CH\(_2\)CO), 1.37 - 1.29 (6H, m, CH\(_3\)), 0.94 - 0.89 (3H, m, CH\(_3\)); \( ^{13}\text{C NMR} \) (125 MHz, CD\(_2\)OD): \( \delta_C \) 176.7 (CH\(_2\)CONH), 175.7 (COCH\(_2\)NH\(_2\)), 45.2 (COCH\(_2\)NH\(_2\)), 40.0 (CH\(_3\)), 39.9 (CH\(_3\)), 37.2 (CH\(_2\)CO), 32.7 (CH\(_2\)CO), 30.0 (CH\(_3\)), 26.9 (CH\(_2\)CO), 23.6 (CH\(_2\)CO), 14.4 (CH\(_3\)); HRMS (ESI): calculated for C\(_{13}\)H\(_{23}\)N\(_3\)NaO\(_2\) [M+Na]\(^+\): 230.1863, found: 230.1867.
2.10 Synthesis of N-(2-(2-aminoacetamido)ethyl) decanamide (12)

![Chemical structure of intermediate 45 and product 12]

Intermediate 45 (benzyl (2-((2-decanamidoethyl)amino)-2-oxoethyl)carbamate) was synthesised according to general method IV from 35 (319 mg, 1.49 mmol), 42 (342 mg, 1.64 mmol), oxalyl chloride (140 µL, 1.64 mmol), dry DMF (31.0 µL, 0.40 mmol) and anhydrous pyridine (256 µL, 3.17 mmol). The crude product was purified by suspending in cold MeOH (2 mL) and filtering the solid, followed by washing of it with diethyl ether. Pure 45 was obtained as a white solid (289 mg, 48 %); Rf = 0.44 (EtOAc : MeOH 9:1); ¹H NMR (400 MHz, CDCl₃): δₓ 7.33 - 7.38 (5H, Ar-H), 6.70 (1H, br s, NH), 5.99 (2H, s, CH₂Ar), 5.36 (1H, br s, NH), 3.85 (2H, J = 6.0 Hz, COCH₂NH₂), 3.41 (4H, s, NHCH₂CH₂NH₂), 2.17 (2H, t, J = 7.5 Hz, CH₂CO), 1.64 – 1.57 (2H, m, CH₂CH₂CO), 1.32 – 1.20 (12H, m, CH₃), 0.88 (3H, t, J = 7.0 Hz, CH₃); ¹³C NMR (125 MHz, CD₃OD): δₐ 176.9 (CH₂CONH), 172.7 (COCH₂NH₂), 159.5 (COO), 138.3 (Ar), 129.6 (Ar), 129.2 (Ar), 129.0 (Ar), 68.1 (CH₂Ar), 45.4 (COCH₂NH₂), 40.5 (CH₂), 40.1 (CH₃), 37.4 (CH₂CO), 33.1 (CH₃), 30.6 (CH₂), 30.5 (2 x CH₂), 30.5 (CH₃), 27.0 (CH₂CH₂CO), 23.7 (CH₂), 14.4 (CH₃); HRMS (ESI): calculate for C₂₂H₃₅N₃NaO₄ [M+Na⁺]: 428.2520, found: 428.2523.

Intermediate 45 (493 mg, 0.49 mmol) was subjected to hydrogenation according to general method II using Pd/C (52.0 mg, 0.05 mmol) to obtain the final compound 12 as a white powder (132 mg, 99 %). Rf = 0.53 (CH₃Cl₂: MeOH 9:1).
2.11 Synthesis of N-(2-(3-aminopropanamido)ethyl) butyramide (13)

Intermediate 47 (benzyl (3-((2-butyramidoethyl)amino)-3-oxopropyl) carbamate) was synthesised according to the general method I from compound 27 (128 mg, 0.89 mmol), Z-β-Ala-OH (46, 200 mg, 0.89 mmol), DIPEA (311 µL, 1.78 mmol) and HATU (441 mg, 1.16 mmol). The crude product was purified by column chromatography with a stepwise gradient (from pure EtOAc to EtOAc : MeOH : CD3OD = 99:1), giving 47 as a white powder (261 mg, 88%); Rf = 0.52 (EtOAc : MeOH 99:1). 1H NMR (500 MHz, CD3OD): δH 7.42 – 7.26 (5H, m, Ar-H), 5.07 (2H, s, CH2Ar), 3.39 (2H, t, J = 6.5 Hz, COCH2CH2NH), 3.25 – 3.23 (4H, m, NHCH2CH2NH), 2.38 (2H, t, J = 6.5 Hz, COCH2CH2NH), 2.16 (2H, t, J = 7.5 Hz, CH2CO), 1.62 (2H, sext, J = 7.5 Hz, CH2CH2), 0.93 (3H, t, J = 7.5 Hz, CH3); 13C NMR (125 MHz, CDCl3): δC 176.6 (CH2CONH), 174.2 (NHCOCCH), 158.8 (COO), 138.4 (Ar), 129.5 (Ar), 129.0 (Ar), 128.9 (Ar), 67.5 (CH2Ar), 40.2 (CH3), 39.9 (CH2), 39.9 (CH2CO), 38.6 (COCH2CH2), 37.5 (COCH2CH2), 20.3 (CH3CH2), 14.1 (CH3); HRMS (ESI): calculated for C17H25N3NaO4 [M+Na]+: 358.1737, found: 358.1736.

Intermediate 47 (200 mg, 0.60 mmol) was subjected to hydrogenation according to general method II using Pd/C (510 mg, 0.48 mmol) to obtain the final compound 13 as a white powder (120 mg, 99%). Rf = 0.07 (CH3Cl: MeOH 3:7)

\[ \text{HO-} \begin{array}{c} \text{O} \\ \text{N} \\ \text{N} \\ \text{O} \end{array} \text{-Ph} \rightarrow \begin{array}{c} \text{O} \\ \text{N} \\ \text{N} \\ \text{O} \\ \text{Ar} \end{array} \text{NH} \]

Intermediate 47 (benzyl (3-((2-butyramidoethyl)amino)-3-oxopropyl) carbamate) was synthesised according to the general method I from compound 27 (128 mg, 0.89 mmol), Z-β-Ala-OH (46, 200 mg, 0.89 mmol), DIPEA (311 µL, 1.78 mmol) and HATU (441 mg, 1.16 mmol). The crude product was purified by column chromatography with a stepwise gradient (from pure EtOAc to EtOAc : MeOH : CD3OD = 99:1), giving 47 as a white powder (261 mg, 88%); Rf = 0.52 (EtOAc : MeOH 99:1). 1H NMR (500 MHz, CD3OD): δH 7.42 – 7.26 (5H, m, Ar-H), 5.07 (2H, s, CH2Ar), 3.39 (2H, t, J = 6.5 Hz, COCH2CH2NH), 3.25 – 3.23 (4H, m, NHCH2CH2NH), 2.38 (2H, t, J = 6.5 Hz, COCH2CH2NH), 2.16 (2H, t, J = 7.5 Hz, CH2CO), 1.62 (2H, sext, J = 7.5 Hz, CH2CH2), 0.93 (3H, t, J = 7.5 Hz, CH3); 13C NMR (125 MHz, CDCl3): δC 176.6 (CH2CONH), 174.2 (NHCOCCH), 158.8 (COO), 138.4 (Ar), 129.5 (Ar), 129.0 (Ar), 128.9 (Ar), 67.5 (CH2Ar), 40.2 (CH3), 39.9 (CH2), 39.9 (CH2CO), 38.6 (COCH2CH2), 37.5 (COCH2CH2), 20.3 (CH3CH2), 14.1 (CH3); HRMS (ESI): calculated for C17H25N3NaO4 [M+Na]+: 358.1737, found: 358.1736.

Intermediate 47 (200 mg, 0.60 mmol) was subjected to hydrogenation according to general method II using Pd/C (510 mg, 0.48 mmol) to obtain the final compound 13 as a white powder (120 mg, 99%). Rf = 0.07 (CH3Cl: MeOH 3:7)
2.12 Synthesis of N-(2-[(3-aminopropanamido)ethyl] heptanamide (14)

![Chemical structure image]

Scheme 125: Preparation of N-(2-[(3-aminopropanamido)ethyl] heptanamide (14).

Intermediate 48 (benzyl (3-((2-heptanamidoethyl)amino)-3-oxopropyl) carbamate) was synthesised according to general method I from compound 31 (153 mg, 0.89 mmol), 46 (200 mg, 0.89 mmol), DIPEA (310 µL, 1.78 mmol) and HATU (441 mg, 1.16 mmol). The crude product was purified by column chromatography with a stepwise gradient (from pure ethyl acetate to EtOAc : methanol 9:1), affording 48 as a white powder (176 mg, 52 %); Rf = 0.07 (pure EtOAc), \( \nu_{max}/cm^{-1} \): 3325, 2925, 2857, 1680, 1638, 1538, 1234, 728, 692; \(^1\)H NMR (500 MHz, CD3OD): \( \delta \) 7.40 – 7.24 (5H, m, Ar-H), 5.05 (2H, s, CH2Ar), 3.37 (2H, t, J = 7.0 Hz, COCH2CH2NH), 3.27 – 3.20 (4H, m, NHCH2CH2NH), 2.43 (2H, t, J = 7.0 Hz, COCH2CH2NH), 2.15 (2H, t, J = 7.5 Hz, CH2CO), 1.63 – 1.51 (2H, m, CH2CH2CO), 1.38 – 1.21 (6H, m, CH3), 0.91 – 0.86 (3H, m, CH3); \(^{13}\)C NMR (125 MHz, CD3OD): \( \delta \) C 176.8 (CH2CONH), 174.2 (NHCOCH2), 159.1 (COO), 138.7 (Ar), 129.5 (Ar), 129.0 (Ar), 128.8 (Ar), 67.5 (CH2Ar), 40.2 (CH3), 39.9 (CH3), 38.6 (COCH2CH2), 37.5 (COCH2CH2) 37.2 (CH2CO), 32.7 (CH3), 30.1 (CH3), 26.9 (CH2CH2CO), 23.6 (CH3), 14.4 (CH3); HRMS (ESI): calculated for C20H31N3NaO4 [M+Na]^+: 400.2207, found: 400.2204.

Intermediate 48 (217 mg, 0.64 mmol) was subjected to hydrogenation according to general method II using Pd/C (36.0 mg, 0.51 mmol) to obtain the final compound 14 as a white powder (100 mg, 91 %).

\( \nu_{max}/cm^{-1} \): 3289, 2923, 2856, 1634, 1553, 1279, 722; \(^1\)H NMR (500 MHz, CD3OD): \( \delta \) 3.30 – 3.26 (4H, m, NHCH2CH2NH), 2.90 (2H, t, J = 7.0 Hz, COCH2CH2NH), 2.35 (2H, t, J = 6.5 Hz, COCH2CH2NH), 2.18 (2H, t, J = 7.5 Hz, CH2CO), 1.64 – 1.57 (2H, m, CH2CH2CO), 1.38 – 1.27 (6H, m, CH3), 0.95 – 0.88 (3H, m, CH3); \(^{13}\)C NMR (125 MHz, CD3OD): \( \delta \) C 176.7 (CH2CONH), 174.8 (NHCOCH2), 40.1 (CH3), 39.9 (CH3), 39.3 (CH2CO), 38.9 (CH2CH2NH), 37.2 (COCH2CH2NH), 32.7 (CH2CH2CH3), 30.0 (CH2), 26.9 (CH2CH2CO), 23.6 (CH3CH2), 14.4 (CH3); HRMS (ESI): calculated for C12H26N3O2 [M+H]^+: 244.2020, found: 244.2020.
2.13 Synthesis of N-(2-(3-aminopropanamido)ethyl) decanamide (15)

Intermediate 49 (benzyl (3-((2-decanamidoethyl)amino)-3-oxopropyl) carbamate) was synthesised according to the general method I from compound 35 (125 mg, 0.58 mmol), 46 (130 mg, 0.58 mmol), DIPEA (202 µL, 1.16 mmol) and HATU (285 mg, 0.75 mmol). The crude product was purified by column chromatography with a stepwise gradient (from pure EtOAc to EtOAc:MeOH 96:4) to afford 49 as a white powder (120 mg, 86 %); Rf = 0.10 (pure EtOAc). $\nu_{\text{max}}$/cm$^{-1}$: 3300, 2923, 2835, 1689, 1644, 1531, 1286, 1238, 1044, 731, 560, 458; $^1$H NMR (300 MHz, CD$_3$OD): $\delta$H 7.41 – 7.25 (5H, m, Ar-H), 5.05 (2H, s, CH$_2$Ar), 3.37 (2H, t, J = 7.0 Hz, COCH$_2$CH$_2$NH), 3.28 – 3.21 (4H, m, NHCH$_2$CH$_2$NH), 2.36 (2H, t, J = 6.5 Hz, COCH$_2$CH$_2$NH), 2.19 – 2.11 (2H, m, CH$_2$CO), 1.64 – 1.50 (2H, m, CH$_2$CH$_2$CO), 1.36 – 1.20 (12H, m, CH$_2$), 0.91 – 0.85 (3H, m, CH$_3$); $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$C 176.8 (CH$_3$CONH), 174.2 (NHCOCH$_3$), 159.1 (COO), 138.7 (Ar), 129.5 (Ar), 129.0 (Ar), 128.8 (Ar), 67.5 (CH$_2$Ar), 40.2 (CH$_2$), 39.9 (CH$_3$), 38.6 (COCH$_2$CH$_2$NH$_2$), 37.5 (COCH$_2$CH$_2$NH$_2$) 37.2 (CH$_2$CO), 32.7 (CH$_2$), 30.1 (CH$_2$), 29.4 (CH$_3$), 29.3 (2 x CH$_3$), 26.9 (CH$_2$CH$_2$CO), 23.6 (CH$_2$), 14.4 (CH$_3$); HRMS (ESI): calculated for C$_{23}$H$_{37}$N$_3$NaO$_4$ [M+Na$^+$]: 442.2676, found: 442.2678.

Intermediate 49 (100 mg, 0.24 mmol) was subjected to hydrogenation according to general method II using Pd/C (20.0 mg, 0.19 mmol) to obtain the final compound 15 as a white powder (68.0 mg, 99 %). Rf = 0.04 (CH$_3$Cl$_2$: MeOH 3:7)

$\nu_{\text{max}}$/cm$^{-1}$: 3293, 2917, 2847, 1628, 1533, 1467, 1247, 722, 522; $^1$H NMR (400 MHz, CD$_3$OD): $\delta$H 3.31 – 3.26 (4H, m, NHCH$_2$CH$_2$NH), 2.92 (2H, t, J = 6.5 Hz, COCH$_2$CH$_2$NH$_2$), 2.37 (2H, t, J = 6.5 Hz, COCH$_2$CH$_2$NH$_2$), 2.19 (2H, t, J = 7.5 Hz, CH$_2$CO), 1.66 – 1.56 (2H, m, CH$_2$CH$_2$CO), 1.37 – 1.27 (12H, m, CH$_2$), 0.91 (3H, t, J = 6.5 Hz, CH$_3$); $^{13}$C NMR (100 MHz, CD$_3$OD): $\delta$C 176.7 (COCH$_2$CH$_3$), 174.6 (CH$_2$CO), 40.1 (CH$_3$), 39.9 (CH$_3$), 38.9 (COCH$_2$CH$_2$NH$_2$), 38.9 (COCH$_2$CH$_2$NH$_2$), 37.2 (CH$_2$CO), 33.1 (CH$_3$), 30.6 (CH$_2$), 30.6 (CH$_2$), 30.5 (CH$_2$), 30.4
Novel chemical probes for the investigation of nonribosomal peptide assembly

(CH$_3$)$_2$, 26.9 (CH$_2$CH$_2$CO), 23.7 (CH$_3$), 14.4 (CH$_2$CH$_2$); HRMS (ESI): calculated for C$_{13}$H$_{22}$N$_3$O$_2$ [M+H]$^+$: 286.2489, found: 286.2490.

2.14 Synthesis of (R)-N-(2-(2-amino-3-hydroxypropanamido)ethyl)heptanamide (16)

**Scheme 145**: Preparation of (R)-N-(2-(2-amino-3-hydroxypropanamido)ethyl)heptanamide (16).

Intermediate 51 (tert-butyl (R)-(1-[(2-heptanamidoethyl)amino]-3-hydroxy-1-oxopropan-2-yl) carbamate) was synthesised according to general method I from 31 (360 mg, 2.09 mmol), 50 (500 mg, 2.09 mmol), DIPEA (728 µL, 4.18 mmol) and HATU (1.00 g, 2.71 mmol). The crude product was purified by column chromatography with a stepwise gradient (from pure EtOAc to EtOAc : MeOH 97:3), affording 51 as a white powder (502 mg, 70 %); R$_f$ = 0.25 (EtOAc : MeOH 98:2). $v_{max}$/cm$^{-1}$: 3295, 2827, 2858, 1635, 1527, 1241, 1169, 620; $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 7.34 (1H, br s, NHCOCH), 6.69 (1H, br s, CONHC$_2$H$_5$), 5.82 – 5.79 (1H, m, NHCOO), 4.15 (1H, br s, COCHNH), 3.93 (1H, dd, $J$ = 11.0 Hz, 3.5 Hz, CH$_2$OH), 3.67 (1H, s, CH$_3$OH), 3.43 – 3.30 (4H, m, NHCH$_2$CH$_2$NH), 2.14 (2H, t, $J$ = 7.0 Hz, CH$_2$CO), 1.61 – 1.52 (2H, m, CH$_2$CH$_2$CO), 1.42 (9H, s, (CH$_3$)$_3$), 1.30 – 1.22 (6H, m, CH$_2$), 0.88 – 0.82 (3H, m, CH$_3$); $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta_c$ 175.0 (CH$_2$CO), 172.3 (COCH), 156.1 (COO), 80.4 (C(CH$_3$)$_3$), 63.1 (CH$_2$OH), 56.2 (COCH), 39.8 (CH$_3$), 39.5 (CH$_2$), 36.7 (CH$_2$CO), 31.6 (CH$_3$), 29.1 (CH$_3$), 28.5 ((CH$_3$)$_2$), 25.7 (CH$_2$CH$_2$CO), 22.6 (CH$_2$), 14.1 (CH$_3$CH$_2$); HRMS (ESI): calculated for C$_{17}$H$_{33}$N$_3$O$_5$ [M+Na]$^+$: 382.2312, found: 382.2312.

TFA (2.0 mL, 6.53 mol) was added to a solution of 51 (116 mg, 0.32 mmol) in dry dichloromethane (2.0 mL). The reaction mixture was stirred at room temperature for 1.5 h before dichloromethane and TFA were removed in vacuo. The crude material was dissolved in water and saturated aqueous NaHCO$_3$ added. The aqueous layer was extracted with a mixture of 2-isopropanol and chloroform (1:4, 5 x 10 mL). The organic phases were dried over MgSO$_4$ and filtered. The solvent was removed in vacuo to afford the final compound 16 as a white powder (70 mg, 84%).
2.15 Synthesis of (S)-N-(2-(2-amino-3-phenylpropanamido)ethyl)heptanamide (17)

Intermediate 53 (benzyl (S)-(1-((2-heptanamidoethyl)amino)-1-oxo-3-phenylpropan-2-yl) carbamate) was synthesised according to general method I from 31 (360 mg, 2.09 mmol), Z-Phe-OH (52, 630 mg, 2.09 mmol), DIPEA (728 µL, 4.18 mmol) and HATU (1.00 g, 2.71 mmol). The crude product was purified by column chromatography with an elution of EtOAc: petroleum ether (3:1), affording 53 as a white powder (250 mg, 66%). \( R_f = 0.32 \) (EtOAc: petroleum ether 3:1); \( \nu_{\text{max}}/\text{cm}^{-1} \): 3295, 2927, 2853, 1686, 1644, 1533, 1259, 1042, 749, 694; \(^1\)H NMR (500 MHz, CDCl\(_3\)): \( \delta \) 7.32 - 7.06 (10H, Ar-H), 6.26 (1H, br s, NH), 5.80 (1H, br s, NH), 5.30 - 5.18 (1H, m, COCHN), 5.01 (2H, dd, \( J = 12.5, 6.5 \) Hz, OCH\(_2\)Ar), 4.28 (1H, dd, \( J = 7.0, 6.5 \) Hz, CHCH\(_2\)), 3.75 - 3.10 (4H, m, NHCH\(_2\)CH\(_2\)NH), 3.06 - 2.93 (2H, m, CHCH\(_2\)), 2.03 (2H, t, \( J = 7.5 \) Hz, CHCO), 1.55 - 1.46 (2H, m, CH\(_2\)CH\(_2\)CO), 1.26 - 1.10 (6H, m, CH\(_3\)), 0.83 - 0.78 (3H, m, CH\(_3\)) \(^1\)C NMR (125 MHz, CDCl\(_3\)): \( \delta \) C 174.1 (CH\(_2\)CONH), 171.7 (COCH), 155.9 (COO), 136.4 (Ar), 136.1 (Ar), 129.3 (Ar), 128.8 (Ar), 128.6 (Ar), 128.3 (Ar), 128.1 (Ar), 127.2 (Ar), 67.2 (OCH\(_2\)Ar), 67.3 (CHCH\(_2\)Ar), 56.5 (COCH), 40.1 (CH\(_3\)), 39.5 (CH\(_3\)), 38.6 (CH\(_2\)CH), 36.7 (CH\(_2\)CO), 31.5 (CH\(_2\)), 29.0 (CH\(_3\)), 25.6 (CH\(_2\)CH\(_2\)CO), 22.5 (CH\(_2\)CH\(_3\)), 14.1 (CH\(_3\)); HRMS (ESI): calculated for C\(_{26}\)H\(_{36}\)N\(_3\)O\(_4\) [M+Na\(^+\)]: 476.2607, found: 476.2609.

Intermediate 53 (70 mg, 0.154 mmol) was then subjected to hydrogenation according to general method II using Pd/C (213 mg, 0.123 mmol) to obtain the final compound 17 as a white powder (49 mg, 100%).
v_{\text{max}}/\text{cm}^{-1}: 2917, 2849, 1722, 1365, 1247, 1142, 845, 716; \textsuperscript{1}H \text NMR (500 MHz, CD_{3}OD): \delta_H 7.32 - 7.19 (5H, m, Ar-H), 3.50 (1H, t, J = 7.0 Hz, CH), 3.25 - 3.15 (4H, m, NHCH_{2}CH_{2}NH), 2.99 (1H, dd, J = 13.5, 6.5 Hz, CHCH_{2}), 2.80 (1H, dd, J = 13.5, 7.5 Hz, CHCH_{2}), 2.15 (2H, t, J = 7.5 Hz, CH_{2}CO), 1.62 - 1.54 (2H, m, CH_{2}CH_{2}CO), 1.36 - 1.26 (6H, m, CH_{3}), 0.91 - 0.89 (3H, m, CH_{3}); \textsuperscript{13}C \text NMR (125 MHz, CDCl_{3}): \delta_C 177.1 (COCH), 176.6 (CH_{2}CO), 138.9 (Ar), 130.4 (Ar), 129.6 (Ar), 127.8 (Ar), 57.9 (CH), 42.5 (CHCH_{2}), 39.9 (CH_{2}), 39.8 (CH_{2}), 37.2 (CH_{2}CO), 32.7 (CH_{2}CH_{2}CH_{2}CO), 30.0 (CH_{2}), 26.9 (CH_{2}CH_{2}CO), 23.6 (CH_{2}), 14.4 (CH_{3}CH_{2}); \textbf{HRMS (ESI)}: calculated for C_{18}H_{30}N_{3}O_{2} [M+H]^+: 319.2020, found: 319.2019; [\alpha]_D^{33} = +8.14 (c = 0.018, CH_{3}OH).
3 Feeding experiments

3.1 General microbiology methods

All glassware and media were prepared and sterilised by autoclaving (Astell) according to reported procedures. Liquid cultures were grown with shaking in Innova 44 incubator/Shaker (New Brunswick scientific), solid cultures were grown in Hearaeus incubator (Thermo). *S. lasaliensis* ACP12(S970A) was grown and maintained as previously described.

Table 15: List and composition of media utilised.

<table>
<thead>
<tr>
<th>Media</th>
<th>Ingredient</th>
</tr>
</thead>
<tbody>
<tr>
<td>M79 media</td>
<td>2.5 g glucose, 2.5 g peptone, 0.5 g yeast extract, 1.5 g NaCl, 2.5 g casamino acids, and 250 mL deionised water (final volume), adjusted to pH 7.0.</td>
</tr>
<tr>
<td>MYM Agar</td>
<td>0.8 g maltose, 0.8 g yeast extract, 2.0 g malt extract, 4.0 g agar, and 200 mL deionised water (final volume), adjusted to pH 7.2.</td>
</tr>
<tr>
<td>MYM Liquid</td>
<td>0.8 g maltose, 0.8 g yeast extract, 2.0 g malt extract, and 200 mL deionised water (final volume), adjusted to pH 7.2.</td>
</tr>
</tbody>
</table>

*S. lasaliensis* ACP12(S970A) strain (100 µL glycerol spore stock) was grown in M79 medium (10 mL) for 3 days, 250 rpm at 30 °C in 50 mL Erlenmeyer flasks with spring. Seed cultures (100 µL) were used to either inoculate MYM liquid cultures (5 mL, in duplicates, in 50 mL Erlenmeyer flasks with spring) or MYM agar plates (5 mL, in duplicates, in 10 mL petri dishes) with varying concentrations (0.04 – 4.0 mM) of probes (3 – 17) dissolved in methanol (100 µL). Liquid cultures were incubated at 30 °C, 250 rpm for 5 days. After the first day of fermentation, the probes were added portionwise (10 µmol dissolved in 10 µL of MeOH per day) to liquid cultures over days 2 - 5 to reach the final concentration, whereas solid cultures on MYM agar already containing the probes at variable concentrations were incubated at 30 °C for 5 days. Both control liquid and solid cultures in the absence of the probes were also prepared (in duplicates). After 5 days, all cultures were extracted with ethyl acetate (10 mL) or methanol (agar plates only, 10 mL). The extracts were filtered and concentrated, and the residues were redissolved in HPLC-grade methanol (500 µL) for mass spectrometry analysis.
3.2 **High-resolution LC-MS analyses of extracts**

HR-ESI-MS analyses of *S. lasaliensis* ACP12 (S970A) strains were performed on a MaXis Impact UHR-TOF (Bruker Daltonics) and on an Orbitrap Fusion with UltiMate 3000 RSLCnano System (Thermo Scientific). % of concentrations indicated in HPLC solvents/conditions are v/v.

UPLC-HR-ESI-MS analyses of extracts on a MaXis Impact UHR-TOF (Bruker Daltonics): samples (5 µL) were injected onto an Acquity UPLC HSS T3 (150 mm x 1.0 mm, 1.8 µm) or Agilent Eclipse C18 (1.8um, 100 mm x 2.1 mm). The mobile phase consisted of a gradient of water and acetonitrile (HPLC grade, each with 0.1 % trifluoroacetic acid). The following solvent (A =0.1% TFA in H₂O, B =0.1% TFA in MeCN) gradient was applied: 10% B 0-2.7 min; 10-100 % B 2.7-42.7 min; 100 % B 42.7-52.7 min; 100-10 % B 52.7-55.7 min; 10 % B 55.7-67.7 min, using an Acquity UPLC HSS T3 column at a flow rate of 0.05 mL/min. Spectra were recorded in positive ionisation mode, scanning from m/z 100 to 3000, with the resolution set at 45K. Selected ion search within 5 ppm was performed.

Further **high resolution analyses** for extracts obtained from N-butyryl to N-decanoyl probes were performed on a Thermo Orbitrap Fusion (Q-OT-qIT, Thermo) instrument. Reversed phase chromatography was used to separate the mixtures prior to MS analysis. Two columns were utilised: an Acclaim PepMap μ-precolumn cartridge 300 μm i.d. x 5 mm 5 μm 100 Å and an Acclaim PepMap RSLC 75 μm x 15 cm 2 μm 100 Å (Thermo Scientific). The columns were installed on an Ultimate 3000 RSLCnano system (Dionex). Mobile phase buffer A was composed of 0.1 % aqueous formic acid and mobile phase B was composed of 100 % acetonitrile containing 0.1 % formic acid. Samples were loaded onto the μ-precolumn equilibrated in 2 % aqueous acetonitrile containing 0.1 % trifluoroacetic acid for 5 min at 10 µL min⁻¹ after which compounds were eluted onto the analytical column following a gradient for which the mobile phase B concentration was increased from 3 % to 90 % over 15 min, then maintained at 90 % B for 5 min, then decreasing to 3 % B over 16 min, followed by a 9 min wash at 3 % B. Eluting cations were converted to gas-phase ions by electrospray ionization and analysed. Survey scans of precursors from 150 to 1500 m/z were performed at 60K resolution (at 200 m/z) with a 5 × 10⁵ ion count target. Tandem MS was performed by isolation at 0.7 Th with the quadrupole, HCD fragmentation with normalized collision energy of 30, and rapid scan MS analysis in the ion trap. The MS² ion count target was set to 10⁴ and the maximum injection time was 35 ms. A filter targeted inclusion mass list was used to select the precursor ions. The dynamic exclusion duration was set to 45 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 5 s cycles, meaning the instrument would continuously perform MS² events until the list of non-excluded precursors diminishes to zero or 5 s, whichever is shorter. Fusion runs were performed with Survey scans of
precursors from 150 to 1500 m/z 60K resolution (at 200 m/z) with a $1 \times 10^6$ ion count target. Tandem MS was performed by isolation at 1.8 Th with the ion-trap, CAD fragmentation with normalized collision energy of 32, and 15 K resolution scan MS analysis in the Orbitrap. MS$^2$ ion count target was set to $4 \times 10^6$ and the max injection time was 50 ms. The dynamic exclusion duration was set to 40 s with a 10 ppm tolerance around the selected precursor and its isotopes.

### 3.3 Capture of peptide intermediates from S. lasaliensis ACP12(S970A) via L-alanine-based chain termination probes 3-6

Table 25: Overview of putative intermediates captured from *S. lasaliensis* ACP12(S970A) via alanine-based probes 3-6 (analysed on a MaXis Impact instrument$^{[a]}$ and on an Orbitrap Fusion instrument$^{[b]}$).

<table>
<thead>
<tr>
<th>Putative intermediate structures</th>
<th>Probe 3$^{[a]}$</th>
<th>Probe 4$^{[a],[b]}$</th>
<th>Probe 5$^{[b]}$</th>
<th>Probe 6$^{[b]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>R$_3$ = CH$_3$</td>
<td>n.d.</td>
<td>✓</td>
<td>✓</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Modest abundance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R$_3$ = (CH$_2$)$_2$CH$_3$</td>
<td></td>
<td>n.d.</td>
<td>✓</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R$_3$ = (CH$_2$)$_5$CH$_3$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R$_3$ = (CH$_2$)$_8$CH$_3$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.d. = Not determined
3.3.1 Putative echinomycin intermediate capture via $(S)$-N-(2-(2-aminopropanamido)ethyl) butyramide probe (4)

![Image of growth plates](image_url)

**Figure 15:** Growth of *S. lasaliensis* ACP12(S970A) in the absence (control) and in the presence of 2.0 mM and 4.0 mM of probe 4 on MYM agar plates.

![Image of mass spectra](image_url)

**Figure 25:** Echinomycin (18) production in *S. lasaliensis* ACP12(S970A) grown on MYM agar.
Figure 3S: HR-MS$^2$ characterisation of probe 4 (Orbitrap Fusion) from the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 4 (4.0 mM on MYM agar).
Figure 4S: Detection and characterisation of putative dipeptide intermediate 54. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of S. lasaliensis ACP12(S970A) grown in the presence of probe 4 (MYM agar, 4.0 mM concentration): extracted ion chromatogram ([M+H]+, top) and MS² fragmentation for 54 (bottom). This species was not found in control samples (data not shown).
3.3.2 Putative echinomycin intermediate capture via (S)-N-(2-(2-aminopropanamido)ethyl) heptanamide probe (5)

Figure 5S: Detection and characterisation of probe 5. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(5970A) grown in the presence of probe 5 (2.0 mM on MYM agar): (A) extracted ion chromatogram ([M+H]+), (B) accurate mass and isotopic distribution and (C) MS² fragmentation for 5.
**Figure 6S:** Detection and characterisation of putative dipeptide intermediate 55. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 5 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]+, top) and MS² fragmentation for 55 (bottom). This species was not found in control samples (data not shown).
Figure 7S: Detection and characterisation of putative tripeptide intermediate 56. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 5 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]+, top) and MS² fragmentation for 56 (bottom). This species was not found in control samples (data not shown).
Figure 8S: Detection and characterisation of putative tetrapetide intermediate 57. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 5 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]+, top) and MS² fragmentation for 57 (bottom, expansion). Further fragments related to 57 (m/z = 244, 226, 173 and 156, as shown in Figure 7S) were found (here not shown). 57 was not found in control samples (data not shown).
3.3.3 Putative echinomycin intermediate capture via (S)-N-(2-(2-aminopropanamido)ethyl) decanamide probe (6)

Figure 9S: Detection and characterisation of probe 6. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasalensis* ACP12(S970A) grown in the presence of probe 6 on MYM agar (2.0 mM): (A) extracted ion chromatogram ([M+H]+), (B) accurate mass and isotopic distribution and (C) MS² fragmentation for 6. Probe 6 proved cytotoxic at concentrations above 1.0 mM.

No peptide intermediates were found/characterised from experiments with 6.
3.4 Capture of peptide intermediates from *S. lasaliensis* ACP12(S970A) via L-valine-based chain termination probes 7-8

Table 35: Overview of putative intermediates captured from *S. lasaliensis* ACP12(S970A) via valine-based probes 7-8 (analysed on a MaXis Impact instrument[^5] and on an Orbitrap Fusion instrument[^6]).

<table>
<thead>
<tr>
<th>Putative intermediate structures</th>
<th>Probe 7[^4][^5]</th>
<th>Probe 8[^6]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R&lt;sub&gt;3&lt;/sub&gt; = (CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>R&lt;sub&gt;3&lt;/sub&gt; = (CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;5&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>modest abundance</td>
<td>modest abundance</td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>traces</td>
<td>low abundance</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure 3" /></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>n.d.</td>
<td>low abundance</td>
</tr>
<tr>
<td><img src="image4.png" alt="Structure 4" /></td>
<td></td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
3.4.1 Putative echinomycin intermediate capture via (S)-2-amino-N-(2-butyramidoethyl)-3-methylbutanamide probe (11)

Figure 10S: Detection and characterisation of probe 7. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of S. lasaliensis ACP12(S970A) grown in the presence of probe 7 (2.0 mM on MYM agar): (A) extracted ion chromatogram ([(M+H)+], (B) accurate mass and isotopic distribution and (C) MS² fragmentation for 7.
Figure 11S: Detection and characterisation of putative dipeptide intermediate 58. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 7 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]^+^, top) and MS^2^ fragmentation for 58 (bottom). This species was not found in control samples (data not shown). A putative tripeptide of m/z 473 was also observed in the same sample (see 59 below) however the very low abundance abundance of MS^2^ fragments for this species (not shown) did not allow his unequivocal identification.
3.4.1 Putative echinomycin intermediate capture via (S)-N-(2-(2-amino-3-methylbutanamido)ethyl)heptanamide probe (8)

**Figure 12S:** Detection and characterisation of probe 8. LC-HRMS analysis (Orbitrap Fusion) of the organic extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 8 (2.0 mM on MYM agar): (A) extracted ion chromatogram ([M+H]^+), (B) accurate mass and isotopic distribution and (C) MS² fragmentation for 8.
Figure 13S: Detection and characterisation of putative dipeptide intermediate 60. LC-HRMS analysis (Orbitrap Fusion) of methanol extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 8 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]⁺, top) and MS² fragmentation for 60 (bottom). This species was not found in control samples (data not shown).
Figure 14S: Detection and characterisation of putative tripeptide intermediate 61. LC-HRMS analysis (Orbitrap Fusion) of methanol extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 8 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]+, top) and MS² fragmentation for 61 (bottom). This species was not found in control samples (data not shown).
**Figure 15S**: Detection and characterisation of putative tetrapeptide intermediate 62. LC-HRMS analysis (Orbitrap Fusion) of methanol extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 8 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]^+\), top) and MS^2 fragmentation for 62 (bottom). This species was not found in control samples (data not shown).
3.5 *Capture of peptide intermediates from S. lasaliensis ACP12(S970A) via L-serine-based chain termination probe 9*

Table 4S: Overview of putative intermediates captured from *S. lasaliensis* ACP12(S970A) via L-Ser-based probe 9 (analysed on an Orbitrap Fusion instrument).

<table>
<thead>
<tr>
<th>Putative intermediate structures</th>
<th>Probe 9 $R_3 = (\text{CH}<em>2)</em>{12}\text{CH}_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Intermediate 1" /></td>
<td>✔️ moderate abundance</td>
</tr>
<tr>
<td><img src="image2" alt="Intermediate 2" /></td>
<td>✔️ low abundance</td>
</tr>
<tr>
<td><img src="image3" alt="Intermediate 3" /></td>
<td>n.d.</td>
</tr>
<tr>
<td><img src="image4" alt="Intermediate 4" /></td>
<td>n.d.</td>
</tr>
</tbody>
</table>
Figure 16S: Detection and characterisation of probe 9. LC-HRMS analysis (Orbitrap Fusion) of the organic extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 9 (2.0 mM on MYM agar): (A) extracted ion chromatogram ([M+H]^+), (B) accurate mass and isotopic distribution and (C) MS^2 fragmentation for 9.
**Figure 17S**: Detection and characterisation of putative dipeptide intermediate 63. LC-HRMS analysis (Orbitrap Fusion) of methanol extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 9 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]⁺, top) and MS² fragmentation for 63 (bottom). This species was not found in control samples (data not shown).
Figure 18S: Detection and characterisation of putative tripeptide intermediate 64. LC-HRMS analysis (Orbitrap Fusion) of methanol extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 9 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]⁺, top) and MS² fragmentation for 64 (bottom). This species was not found in control samples (data not shown).
3.6 Capture of peptide intermediates from *S. lasaliensis* ACP12(S970A) via glycine-based chain termination probes 10-12

Table 55: Overview of putative intermediates captured from *S. lasaliensis* ACP12(S970A) via glycine-based probes 10-12 (analysed on an Orbitrap Fusion instrument).

<table>
<thead>
<tr>
<th>Putative intermediate structures</th>
<th>Probe 10 $R_3 = (\text{CH}_2)_2\text{CH}_3$</th>
<th>Probe 11 $R_3 = (\text{CH}_2)_5\text{CH}_3$</th>
<th>Probe 12 $R_3 = (\text{CH}_2)_8\text{CH}_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Chemical structure 1]</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>![Chemical structure 2]</td>
<td>✓</td>
<td>✓</td>
<td>Low abundance</td>
</tr>
<tr>
<td>![Chemical structure 3]</td>
<td>✓</td>
<td>✓</td>
<td>n.d.</td>
</tr>
<tr>
<td>![Chemical structure 4]</td>
<td>✓</td>
<td>✓</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
3.6.1 Putative echinomycin intermediate capture via 2-(2-aminoacetamido)ethyl) butyramide probe (10)

Figure 19S: LC-HRMS characterisation of probe 10: accurate mass isotopic distribution ([(M+H)⁺, top] and MS² fragmentation (bottom).
Novel chemical probes for the investigation of nonribosomal peptide assembly

Figure 20S: Detection and characterisation of a putative dipeptide intermediate (65) from echinomycin biosynthesis. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of S. lasaliensis ACP12(S970A) grown in the presence of probe 10 (liquid culture, final concentration 4.0 mM): (A) extracted ion chromatogram ([M+H]+), (B) accurate mass and isotopic distribution and (C) MS² fragmentation for 65. This species was not found in control samples (data not shown).
Figure 21S: Detection and characterisation of putative tripeptide intermediate 66. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 10 (agar plate, 2.0 mM concentration). Top: extracted ion chromatogram ([M+H]^+) and MS^2 fragmentation for 66 (bottom). This species was not found in control samples (data not shown).
Figure 22S: Detection and characterisation of putative tetrapeptide intermediate 67. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 10 (agar plate, 2.0 mM concentration). Top: extracted ion chromatogram ([M+H]+) and MS² fragmentation for 67 (bottom, expansion). Further fragments related to 67 (m/z= 226, 188, 170, 131 and 114, as shown in Figure 21S) were found (here not shown). 67 could not be found in control samples (data not shown).
Figure 23S: Detection and characterisation of putative pentapeptide intermediate 68. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 10 (agar plate, 2.0 mM concentration). Top: extracted ion chromatogram ([M+H]$^+$) and MS$^2$ fragmentation for 68 (bottom, expansion). Further fragments related to 68 (m/z= 226, 188, 170, 131 and 114, as shown in Figure 21S) were found (here not shown). 68 could not be found in control samples (data not shown).
3.6.2 Putative echinomycin intermediate capture via \( N-(2-(2\text{-aminoacetamido})\text{ethyl}) \) heptanamide probe (11)

**Figure 245:** Detection and characterisation of probe 11. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of \( S. \) lasaliensis ACP12(S970A) grown in the presence of probe 11 (liquid culture, final concentration 4.0 mM): (A) extracted ion chromatogram ([M+H]\(^{+}\)), (B) accurate mass and isotopic distribution and (C) MS\(^{2}\) fragmentation for 11.
Figure 25S: Detection and characterisation of putative dipeptide intermediate 69. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of S. lasaliensis ACP12(S970A) grown in the presence of probe 11 (liquid culture, final concentration 4.0 mM): (A) extracted ion chromatogram ([M+H]^+), (B) accurate mass and isotopic distribution and (C) MS^2 fragmentation for 69. This species was not found in control samples (data not shown).
Figure 26S: Detection and characterisation of putative tripeptide intermediate 70. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of S. lasaliensis ACP12(S970A) grown in the presence of probe 11 (liquid culture, final concentration 4.0 mM): (A) extracted ion chromatogram ([M+H]+) and (B) MS² fragmentation for 70. This species was not found in control samples (data not shown).
Figure 27S: Detection and characterisation of the putative tetrapeptide 71. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 11 (on MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]$^+$, top) and MS$^2$ fragmentation for 71 (bottom, expansion). 71 could not be found in control samples (data not shown).
3.6.3 Putative echinomycin intermediate capture via N-(2-(2-aminoacetamido)ethyl) decanamide probe (12)

**Figure 285:** Detection and characterisation of probe 12. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 12 (0.4 mM on MYM agar): (A) extracted ion chromatogram ([M+H]^+), (B) accurate mass and isotopic distribution and (C) MS^2 fragmentation for 12.

No peptide intermediates were found/characterised from experiments with 12.

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3.7 Capture of peptide intermediates from *S. lasaliensis* ACP12(S970A) via β-alanine-based chain termination probes 13-15

Table 6S: Overview of putative intermediates captured from *S. lasaliensis* ACP12(S970A) via β-alanine-based probes 13-15 (analysed on a MaXis Impact instrument\[a\] and on an Orbitrap Fusion instrument\[b\]).

<table>
<thead>
<tr>
<th>Putative intermediate structures</th>
<th>Probe 13 [^{[a],[b]}]</th>
<th>Probe 14 [^{[a],[b]}]</th>
<th>Probe 15 [^{[a],[b]}]</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Putative intermediate structure 1" /></td>
<td><img src="image1" alt="Putative intermediate structure 1" /></td>
<td><img src="image1" alt="Putative intermediate structure 1" /></td>
<td><img src="image1" alt="Putative intermediate structure 1" /></td>
</tr>
<tr>
<td><img src="image2" alt="Putative intermediate structure 2" /></td>
<td><img src="image2" alt="Putative intermediate structure 2" /></td>
<td><img src="image2" alt="Putative intermediate structure 2" /></td>
<td><img src="image2" alt="Putative intermediate structure 2" /></td>
</tr>
<tr>
<td><img src="image3" alt="Putative intermediate structure 3" /></td>
<td><img src="image3" alt="Putative intermediate structure 3" /></td>
<td><img src="image3" alt="Putative intermediate structure 3" /></td>
<td><img src="image3" alt="Putative intermediate structure 3" /></td>
</tr>
<tr>
<td><img src="image4" alt="Putative intermediate structure 4" /></td>
<td><img src="image4" alt="Putative intermediate structure 4" /></td>
<td><img src="image4" alt="Putative intermediate structure 4" /></td>
<td><img src="image4" alt="Putative intermediate structure 4" /></td>
</tr>
</tbody>
</table>

\(^{[a]}\) R\(_3\) = (CH\(_2\))\(_2\)CH\(_3\)

\(^{[b]}\) R\(_3\) = (CH\(_2\))\(_3\)CH\(_3\)
3.7.1 Putative echinomycin intermediate capture via N-(2-acetamidoethyl)-3-aminopropanamide probe (13)

![Diagram of probe 13 and its mass spectra](image)

**Figure 29S**: Detection and characterisation of probe 13. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 13 (2.0 mM on MYM agar): (A) extracted ion chromatogram ([M+H]⁺), (B) accurate mass and isotopic distribution and (C) MS² fragmentation for 13.
Figure 30S: Detection and characterisation of putative dipeptide intermediate 72. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of S. lasaliensis ACP12(S970A) grown in the presence of probe 13 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]⁺, top), accurate mass isotopic distribution (middle), and MS² fragmentation for 72 (bottom). This species was not found in control samples (data not shown).
Figure 31S: Detection and characterisation of putative tripeptide intermediate 73. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 13 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]^+^, top) and MS^2^ fragmentation for 73 (bottom). This species was not found in control samples (data not shown).
Figure 32S: Detection and characterisation of putative tetrapeptide intermediate 74. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of S. lasaliensis ACP12(S970A) grown in the presence of probe 13 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]^+ , top) and MS^2 fragmentation for 74 (bottom). This species was not found in control samples (data not shown).
3.7.2 Putative echinomycin intermediate capture via 5-N-(2-(3-aminopropanamido)ethyl)heptanamide probe (14)

**Figure 335:** Detection and characterisation of probe 14. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 14 (2.0 mM on MYM agar): (A) extracted ion chromatogram ([M+H]^+), (B) accurate mass and isotopic distribution and (C) MS² fragmentation for 14.
Figure 34S: Detection and characterisation of putative dipeptide intermediate 75. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of S. lasaliensis ACP12(S970A) grown in the presence of probe 14 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]⁺, top) and MS² fragmentation for 75 (bottom). This species was not found in control samples (data not shown).
Figure 35S: Detection and characterisation of putative tripeptide intermediate 76. LC-HRMS analysis (Orbitrap Fusion) of the ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 14 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]^+^, top) and MS^2^ fragmentation for 76 (bottom). 76 was not detected in control samples (data not shown).
3.7.3  Putative echinomycin intermediate capture via N-(2-(3-aminopropanamido)ethyl) decanamide probe (15)

Figure 36S: Growth of *S. lasaliensis* ACP12(S970A) in the absence (control) and in the presence of 2.0 mM probe 15 on MYM agar plates. Oppositely to other N-decanoyl probes, 15 is not cytotoxic at concentrations above 1.0 mM.

Figure 37S: Characterisation of probe 15 from ethyl acetate extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 15 (MYM agar, 2.0 mM concentration): accurate mass isotopic (for [M+H]^+^, top) and HR-MS^2^ fragmentation (bottom).

No peptide intermediates were found/characterised from experiments with 15.
3.8 *Capture of peptide intermediates from S. lasaliensis ACP12(S970A) via D-Serine-based chain termination probe 16*

Table 7S: Overview of putative intermediates captured from *S. lasaliensis* ACP12(S970A) via D-Ser-based probe 16 (analysed on an Orbitrap Fusion instrument).

<table>
<thead>
<tr>
<th>Putative intermediate structures</th>
<th>Probe 16</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Intermediate 1" /></td>
<td><img src="image2" alt="Checkmark" /> low abundance</td>
</tr>
<tr>
<td><img src="image3" alt="Intermediate 2" /></td>
<td>n.d.</td>
</tr>
<tr>
<td><img src="image4" alt="Intermediate 3" /></td>
<td>n.d.</td>
</tr>
<tr>
<td><img src="image5" alt="Intermediate 4" /></td>
<td>n.d.</td>
</tr>
</tbody>
</table>
Figure 38: Detection and characterisation of probe 16. LC-HRMS analysis (Orbitrap Fusion) of the organic extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 16 (2.0 mM on MYM agar): extracted ion chromatogram ([M+H]^+_, top), and accurate MS^2_ fragmentation (bottom).
Figure 39S: Detection and characterisation of putative dipeptide intermediate 77. LC-HRMS analysis (Orbitrap Fusion) of methanol extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 16 (MYM agar, 2.0 mM concentration): extracted ion chromatogram ([M+H]⁺, top) and MS² fragmentation for 77 (bottom). This species was not found in control samples (data not shown). The stereochemistry of 77 is currently under investigation.
3.9 Capture of peptide intermediates from *S. lasaliensis* ACP12(S970A) via L-phenylalanine-based chain termination probe 17

**Figure 40S**: Characterisation of probe 17 from organic extracts of *S. lasaliensis* ACP12(S970A) grown in the presence of probe 18 (MYM agar, 2.0 mM concentration): extracted ion chromatogram (for [M+H]$^+$, top) and HR-MS$^2$ fragmentation (bottom).

No peptide intermediates were consistently found/characterised from experiments with 17.

4 Literature

5 NMR spectra of probes 3-17

5.1 $^1$H- and $^{13}$C-NMR of probe 3
5.2 $^1$H- and $^{13}$C-NMR of probe 4
5.3 $^1H$- and $^{13}C$-NMR of probe 5
5.4 $^1$H- and $^{13}$C-NMR of probe 6
5.5 \textit{\textsuperscript{1}H- and \textsuperscript{13}C-NMR of probe 7}

![NMR spectra of probe 7]

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5.6 $^1$H- and $^{13}$C-NMR of probe 8
5.7 $^1$H- and $^{13}$C-NMR of probe 9
5.8 $^1$H- and $^{13}$C-NMR of probe 10
5.9 \(^{1}H\)- and \(^{13}C\)-NMR of probe 11
5.10 $^1$H- and $^{13}$C-NMR of probe 12
5.11 $^1H$- and $^{13}C$-NMR of probe 13
5.12 $^1$H- and $^{13}$C-NMR of probe 14
5.13 \(^1\text{H- and }^{13}\text{C-NMR of probe 15}

\[
\begin{align*}
\text{15} & \quad \text{O} \\
& \quad \text{N} \quad \text{H} \\
& \quad \text{N} \quad \text{H} \quad \text{O} \\
& \quad \text{NH}_2
\end{align*}
\]

\[
\begin{array}{cccccccccccccccccccc}
\text{δ (ppm)} & 0.89 & 0.91 & 0.93 & 1.32 & 1.59 & 1.61 & 1.62 & 2.17 & 2.19 & 2.21 & 2.35 & 2.37 & 2.38 & 2.91 & 2.92 & 2.94 & 3.29 & 4.87 \\
\text{ resonate in} & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4 & \text{MeOH-d}_4
\end{array}
\]
5.14 $^1$H- and $^{13}$C-NMR of probe 16

[Chemical diagram and NMR spectra]
5.15 $^1$H- and $^{13}$C-NMR of probe 17

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